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PCT / SE 2004 / 001883

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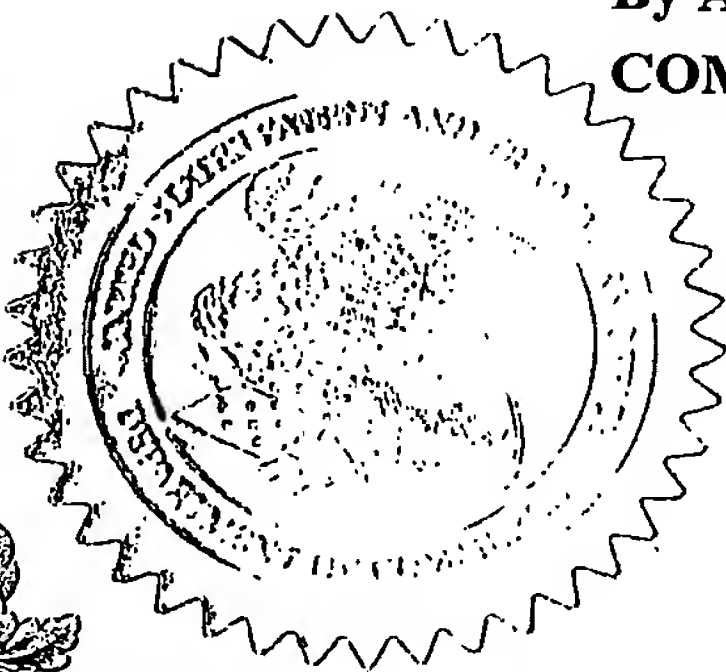
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APPLICATION NUMBER: 60/540,296

FILING DATE: *January 30, 2004*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS



P. SWAIN
Certifying Officer

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PTO/SB/16 (10-01)
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET Pag 1 of 1

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Expr ss Mail Label No.					
INVENTOR(S)					
Given Name (first and middle (if any))		Family Name or Surname		Residence (City and either State or Foreign Country)	
Thomas Maude		BENGTTSSON WIKSTRÖM		Göteborg, SWEDEN Göteborg, SWEDEN	
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
A TEST KIT FOR DETECTING PERIODONTAL DISEASE					
CORRESPONDENCE ADDRESS					
Direct all correspondence to:					
<input checked="" type="checkbox"/> Customer Number		30593		→ 30593	
OR Type Customer Number here					
<input checked="" type="checkbox"/> Firm or Individual Name		HARNES, DICKEY & PIERCE, P.L.C.			
Address		P.O. Box 8910			
Address					
City		Reston	State	VA	ZIP 20195
Country		USA	Telephone	(703) 668-8000	Fax (703) 668-8200
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		29		<input type="checkbox"/> CD(s), Number	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets		1		<input type="checkbox"/> Other (specify)	
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.					
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees					
FILING FEE AMOUNT (\$)					
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any deficiency fees or credit any overpayment to Deposit Account Number: 08-0750 \$160.00					
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,
SIGNATURE

Date

January 30, 2004

TYPED or PRINTED NAME Donald J. Daley

REGISTRATION NO.
(if appropriate)

34,313

TELEPHONE 703-668-8030

Docket Number

10400-000091/US

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT


This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

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FEE TRANSMITTAL for FY 2003		Complete If Known																																																																																																																																																																	
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ADDITIONAL FEES</div> <table border="1" style="width: 100%; border-collapse: collapse;"><thead><tr><th>Fee Code</th><th>Large Entity Fee (\$)</th><th>Small Entity Fee (\$)</th><th>Fee Description</th><th>Fee Paid</th></tr></thead><tbody><tr><td>1051</td><td>130</td><td>2051 65</td><td>Surcharge - late filing fee or oath</td><td></td></tr><tr><td>1052</td><td>50</td><td>2052 25</td><td>Surcharge - late provisional filing fee or cover sheet</td><td></td></tr><tr><td>1053</td><td>1053</td><td>1053 130</td><td>Non-English specification</td><td></td></tr><tr><td>1812</td><td>2,520</td><td>1812 2,520</td><td>For filing a request for reexamination</td><td></td></tr><tr><td>1804</td><td>920*</td><td>1804 920*</td><td>Requesting publication of SIR prior to Examiner action</td><td></td></tr><tr><td>1805</td><td>1,840*</td><td>1805 1,840*</td><td>Requesting publication of SIR after Examiner action</td><td></td></tr><tr><td>1251</td><td>110</td><td>2251 55</td><td>Extension for reply within first month</td><td></td></tr><tr><td>1252</td><td>420</td><td>2252 210</td><td>Extension for reply within second month</td><td></td></tr><tr><td>1253</td><td>950</td><td>2253 475</td><td>Extension for reply within third month</td><td></td></tr><tr><td>1254</td><td>1,480</td><td>2254 740</td><td>Extension for reply within fourth month</td><td></td></tr><tr><td>1255</td><td>2,010</td><td>2255 1,005</td><td>Extension for reply within fifth month</td><td></td></tr><tr><td>1401</td><td>330</td><td>2401 165</td><td>Notice of Appeal</td><td></td></tr><tr><td>1402</td><td>330</td><td>2402 165</td><td>Filing a brief in support of an appeal</td><td></td></tr><tr><td>1403</td><td>290</td><td>2403 145</td><td>Request for oral hearing</td><td></td></tr><tr><td>1451</td><td>1,510</td><td>1451 1,510</td><td>Petition to institute a public use proceeding</td><td></td></tr><tr><td>1452</td><td>110</td><td>2452 55</td><td>Petition to revive - unavoidable</td><td></td></tr><tr><td>1453</td><td>1,330</td><td>2453 665</td><td>Petition to revive - unintentional</td><td></td></tr><tr><td>1501</td><td>1,330</td><td>2501 665</td><td>Utility issue fee (or reissue)</td><td></td></tr><tr><td>1502</td><td>480</td><td>2502 240</td><td>Design issue fee</td><td></td></tr><tr><td>1503</td><td>840</td><td>2503 320</td><td>Plant issue fee</td><td></td></tr><tr><td>1460</td><td>130</td><td>1460 130</td><td>Petitions to the Commissioner</td><td></td></tr><tr><td>1807</td><td>50</td><td>1807 50</td><td>Processing fee under 37 CFR 1.17 (q)</td><td></td></tr><tr><td>1806</td><td>180</td><td>1806 180</td><td>Submission of Information Disclosure Stmt</td><td></td></tr><tr><td>8021</td><td>40</td><td>8021 40</td><td>Recording each patent assignment per property (times number of properties)</td><td></td></tr><tr><td>1809</td><td>770</td><td>2809 385</td><td>Filing a submission after final rejection (37 CFR § 1.129(a))</td><td></td></tr><tr><td>1810</td><td>770</td><td>2810 385</td><td>For each additional invention to be examined (37 CFR § 1.129(b))</td><td></td></tr><tr><td>1801</td><td>770</td><td>2801 385</td><td>Request for Continued Examination (RCE)</td><td></td></tr><tr><td>1802</td><td>900</td><td>1802 900</td><td>Request for expedited examination of a design application</td><td></td></tr><tr><td colspan="5">Other fee (specify) _____</td></tr><tr><td colspan="5">*Reduced by Basic Filing Fee Paid</td></tr><tr><td colspan="4" style="text-align: right;">SUBTOTAL (3)</td><td style="text-align: center;">(\$)</td></tr></tbody></table>		Fee Code	Large Entity Fee (\$)	Small Entity Fee (\$)	Fee Description	Fee Paid	1051	130	2051 65	Surcharge - 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SUBMITTED BY		Complete (if applicable)	
Name (Print/Type)	Donald J. Daley	Registration No. Attorney/Agent	34,313
Signature		Telephone	703-668-8000
		Date	January 30, 2004

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A TEST KIT FOR DETECTING PERIODONTAL DISEASE

Technical field

The present invention relates to a test kit for detecting periodontal disease. The present invention further relates to a method for the diagnosis and prediction
5 of risk for progress of periodontal disease.

Technical background

Periodontitis affects approximately 7-15% of the adults in the western world, making it one of our most
10 common diseases. It is a multifactor disease where the presence of pathogenic bacteria in a pocket between tooth and gum is a necessary but not sufficient criterion. The host inflammatory and immune system also play a crucial role in the development of the disease. The progression
15 of periodontitis is thought to be a chronic inflammatory response to subgingival bacteria, resulting in a destruction of the tooth's supportive tissues. It is cyclic in its behaviour and may remain unnoticed in its early phase.

20 The destructive process is thought to be the result of a complex interaction between the defence system of the host and specific bacterial species in the periodontal pocket. Pathogenic bacteria involved in the appearance and progression of periodontal disease include, but
25 are not limited to, *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*), *Bacteroides forsythus* (now also called *Tannerella forsythensis*), *Actinobacillus actinomy-*
cetemcomitans, *Triponea denticola* and *Prevotella inter-*
media.

30 In later years the importance of the bacteria's virulence products (mainly toxins and enzymes) has been widely studied and is believed to play a major role in the pathogenesis (Eley and Cox (2003)). Bacteria are thought to go through different growth phases and during
35 these phases to be more or less destructive in the perio-

dontal pocket. Active bacteria produce virulence products to help its survival and nutrition in the periodontal pocket. During periods of elevated bacterial activity those virulence products contribute to the destruction of the tooth's supportive tissues and the reduction in effectiveness of the hosts' defence systems.

Today dentists assess periodontitis by measuring the probing depth of the periodontal pocket, examining x-ray images of the tooth attachment to the alveolar bone and refer to bleeding on probing. Risk factors include smoking habits, stress and a family history of periodontitis. The method relies heavily on the subjective expertise of the dentist. Probing depth is only a measurement of the historical attachment loss, thus giving little help in the actual occurrence of Periodontitis or the future progression thereof. Bleeding on probing could indicate a healing process instead of a destructive one.

Occasionally a microbiological sample is taken and sent of to a lab for analysis either by cultivation or DNA-techniques (Checkerboard DNA-DNA hybridization technique developed by Socransky (1994)). However, the answer is obtained in about a week's time and only shows the presence of certain bacteria, which not necessarily indicates periodontitis.

Vast amounts of work has been done to find a chemical compound, mainly a protein, such as an an enzyme or a cytokine, in fluids from the oral cavity of a patient, such as gingival crevicular fluid (GCF), that can diagnose or predict the progression of periodontitis.

Up until now there have been a number of tests and assays developed (Armitage (2003)). These assays have been dedicated to identifying bacteria, bacterial virulence products or host proteins.

Host derived proteins that have been investigated for their diagnostic or prognostic value in periodontitis include mainly products from the human inflammatory system. The role of these proteins is to orchestrate the in-

inflammatory and immune response, remodelling of the tissue or to help in the killing of the invading bacteria.

The most studied host derived proteins intended for diagnosing periodontitis include the natural serine proteases (cathepsin G, azurocidin, proteinase 3, elastase), collagenase, aminotransferases (US Patents nos 4,981,787, 5,834,226 and 4,801,535), alkaline phosphatase, β -glucuronidase (US Patent no 6,277,587), dipeptidyl peptidase, neutrophil gelatinase-related lipocalin (US Patent no 5,866,432), matrix metalloproteinases (US Patents nos 5,736,341 and 6,280,687) and cytokines such as interleukins (especially IL-1 β (US Patent no 5,328,829), IL-6 and IL-8) and inflammatory mediators such as prostaglandin E₂ and tumour necrosis factor- α (TNF- α).

Matrix metalloproteins (MMP's) have been suggested as a marker for periodontal disease. US Patent no 5,736,341 discloses detection of the presence of MMP-8, US patent no 5,866,432 discloses detection of the presence of neutrophil gelatinase-related lipocalin and US patent no 6,280,687 discloses the presence of MMP-13. These three patents suggest a rapid chair side test based on the immunochromatographic principle for the diagnosis and progression prediction of periodontitis. However, in the information paper from the American Association of Periodontology by Oringer (2002) it is argued that additional studies are needed in order to verify the roll of MMP's in the progression of periodontal disease.

US Patent no 6,406,873 claim that two inflammatory mediators (plasminogen activator inhibitor 2 and tissue plasminogen activator) alone or in combination can diagnose periodontitis.

US Patent no 5,248,595 describes a method to simultaneously analyse up to three different periodontal pathogens.

Jin et al. (1999) have been investigating the correlation between presence of periodontal pathogens i.e.

bacteria, by using DNA probing methods, in the periodontal pocket and elastase in the gingival crevice fluid (GCF).

Nisengard et al. (1992) have described a rapid latex agglutination test for the presences of *P. gingivalis*, *A. actinomycetemcomitans* and *P. Intermedia*.

Lamster et al (1994) have investigated clinical attachment loss and it's correlation to β -glucuronidase from human leukocytes.

Eley and Cox (1996) have developed a chair-side test based on enzyme substrate specific to the gingipain from *P. gingivalis*.

Up to date, many methods for assessing periodontal disease activity have been developed. However, none of these above mentioned methods provide an specific and sensitive enough assay to diagnose the periodontitis and the destructive pattern thereof. One consequence of un-specific methods is that several patients will be treated without actually having periodontitis. Clinical observations such as probing depth are not reliable enough because deep pockets do not necessarily harbour an ongoing inflammation, radiographic evaluations have to be combined with detailed clinical observations in order to give an accurate diagnosis and the mere presences of pathogenic bacteria in the periodontal pocket do not accurately reflect disease activity. Moreover the so far developed diagnoses based on enzymatic methods for host or bacteria derived proteins have not been sufficiently specific due to the fact that an enzyme substrate can be cleaved by a multitude of different enzymes.

Different cytokines have also been studied but no rapid and specific tests have been designed.

Thus the dentist is in need of a chair side test kit that preferably:

- is rapid, yielding results within a few minutes.
- requires a minimum amount of time and work effort.

- is robust and can be treated rough in the clinic's environment.
- provides easily interpreted results.
- have a long shelf-life in room temperature or in a fridge.
- fits the tray at the dental clinic, is environmentally friendly and provides good patient information material.

10 Summary of the invention

One object for the present invention is to overcome the drawbacks of the prior art and to provide a test kit that meets the present needs of the dentists. More specifically, the present invention aims to provide a test kit and a method for detecting periodontal diseases that is specific, sensitive and easy to use.

The inventors of the present invention have found that a method comprising the detection of the co-existence of a substance originating from bacteria and a substance originating from the human immune of inflammatory system can be used for this purpose.

The present invention can be used to help dentists and dental hygienists to provide their patients with a more efficient treatment of periodontal disease. The test kit and the method according to the invention may be used for screening suspected teeth, to follow up previous treatment, to decide the most appropriate form of treatment, to choose the right kind of antibiotics and to communicate to the patient the importance of good everyday dental hygiene.

In a first aspect, the present invention relates to a test kit for diagnosing periodontal disease in a patient by analysing a sample from the oral cavity of the patient, said kit comprising a first detection assay for detecting a first substance originating from bacteria and a second detection assay from detection of a second sub-

stance originating from the immune or inflammatory system of the patient.

Preferably, the present invention relates to a test kit for diagnosing periodontal disease in a patient by
 5 analysing a sample from the oral cavity of the patient, said kit comprising at least a first detection assay comprising at least a first affinity ligand having a binding site for binding of a first substance originating from
 10 bacteria, and a second detection assay comprising at least a second affinity ligand having a binding site for binding of a second substance originating from the immune or inflammatory system of the patient.

Preferably, said patient is a mammal, most preferably a human.

15 The result from said first detection assay in combination with the result from said second detection assay is used to detect periodontal disease.

Preferably said first substance is a protein produced by said bacteria. Preferably said protein is a
 20 bacterial virulence product, more preferably an enzyme or a toxin. Preferred enzymes are proteases, such as arg-gingipain from *Porphyromonas gingivalis* and the 48 kDa protease from *Bacteroides forsythus*. An example of an advantageous toxin is the leukotoxin from *Actinobacillus*
 25 *actinomycetemcomitans*.

The second substance is preferably a leukocyte, a cytokine or a human inflammatory mediator. Preferred leukocytes are the natural serine proteases, more preferably a human neutrophil elastase. Preferred cytokines, are in-
 30 terleukin-1 β , interleukin-6 and interleukin-8. An example of an advantageous inflammatory mediator is tumour necrosis factor- α .

The co-existence of both a first substance of bacterial origin and a second substance as defined above,
 35 preferably human neutrophil elastase, should indicate both active bacteria and an active immune or inflammatory system, and this indicates periodontal destruction.

It is clear that even though it is well established in the scientific community that periodontal disease is a multi-factorial disease no one has yet developed a test to analyse both bacterial and host derived products in a sample.

The test kit may also comprise a third detection assay comprising at least a third affinity ligand having a binding site for binding of a third substance originating from bacteria or from the immune or inflammatory system of the patient as defined above, preferably from bacteria, and may in some embodiments even comprise further detection assays for binding of additional substances.

Preferably, the first affinity ligand is an antibody exhibiting selective binding of said first substance, and the second affinity ligand is an antibody exhibiting selective binding of said second substance.

The use of antibodies is advantageous over other methods (e.g. enzymatic methods) since antibodies, once developed and tested for cross-reactivity, are highly specific for their target and are able to detect chemical substances other than enzymes, e.g. toxins and cytokines. Furthermore, methods for development of new antibodies against new antigens are well known to those skilled in the art.

Preferably said detection assays in the test kit according to the present invention comprises immunochromatographic assays.

Among the advantages of using immunochromatographic assays/methods is that they are easily produced and used, have a long shelf-life, yields a quick answer and can be designed to be very specific for the substances intended to be detected.

Said test kit further preferably comprises a support provided with a sample reservoir for receiving a sample, wherein said first and second detection assays are arranged on said support in contact with said sample reser-

voir, directly or via a removably arranged separating means which separates said sample reservoir from said detection assays. Said kit may also comprise additional buffers, preferably in a buffer reservoir separate from
 5 said sample reservoir, for dilution and adaptation of said sample for said detection assays, and at least one sampling device for obtaining the sample.

The individual detection assays comprised in the test kit according to the present invention may be pro-
 10 vided together or separately. In the case where the detection assays are sold separately, samples of GCF etc taken concomitantly are analysed separately on each assay, and the results are combined for the diagnosis of periodontal disease.

15 The test kit and the method according to the present invention provides for a chair-side test for periodontal disease, wherein the dentist or a dental hygienist takes e.g. a GCF-sample from a periodontal pocket. The sample is analysed by the assays provided in the test kit and
 20 the results from these assays is judged according to pre-defined criteria to evaluate the occurrence of ongoing periodontal disease.

In a second aspect, the present invention relates to a method for diagnosing periodontal diseases and/or
 25 predicting the risk for progress of said diseases, said method comprising analyzing a sample from the oral cavity of a patient for the presence of at least a first substance originating from bacteria and the presence of a second substance originating from the immune system or
 30 inflammatory system of the patient, the first and second substances being as defined above.

Furthermore, the diagnostic method according to the present invention may also comprise a method to detect the presence of additional substances as above.

35 Preferably, the methods according to the present invention comprises using a first antibody exhibiting selective binding of said first substance and wherein said

second method comprises using a second antibody exhibiting selective binding of said second substance.

More preferably least one of said first and second methods comprises using an immunochromatographic assay.

5

Brief description of the drawing

Figure 1 shows an embodiment of a test kit wherein two immunochromatographic assays are arranged on a support equipped with a sample reservoir.

10

Detailed description of the invention

The present invention relates to a test kit for detecting periodontal disease in a patient by analysing a sample from the oral cavity of the patient, wherein said
15 kit at least comprises a first detection assay comprising at least a first affinity ligand having a binding site for binding of a first substance originating from bacteria, and a second detection assay comprising at least a
20 second affinity ligand having binding site for binding a second substance originating from the immune or inflammatory system of the patient.

Preferably, said sample from the oral cavity of a patient is gingival crevicular fluid, peri-implant sulcus fluid, saliva or a mouth rinse sample.

25 Gingival crevicular fluid (GCF) is a fluid that flows from the periodontal pocket into the oral cavity. In case of inflammation, GCF contains inflammatory cells, bacteria and their by-products respectively and its contents may be used as a marker for destructive periodontitis. Collecting the GCF is a minimally invasive procedure
30 and the fluid provides a quantitative source of biochemical indicators that reflects the response of the patient as well as the bacterial challenge.

Peri-implant sulcus fluid is the GCF-equivalent in
35 the case a tooth is replaced with a dental implant, i.e. a fluid that flows from the site of the implant into the oral cavity.

GCF and peri-implant sulcus fluid is preferred if a site specific detection of the first and second substances is wanted, since distinct samples can be obtained from each examined tooth surface.

5 Saliva or mouth rinse samples may be used to obtain a detection or diagnosis that not is site specific.

As used here, the term "periodontal disease" and periodontitis shall be interpreted in its broadest sense to encompass such diseases as periodontitis, peri-
10 implantitis (wherein the tissue supporting the implant is disintegrated), and other forms of periodontal disease as defined at the 1999 International workshop for classification of periodontal diseases and conditions.

Periodontal pathogenic bacteria, such as *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitans*, *Tritonema denticola* and *Prevotella intermedia* can be present in the oral cavity and in the pocket between a tooth and the healthy gum without this leading to the progression of periodontal disease. The
20 bacteria need certain growth conditions and presences of nutrients to become active. An active bacteria produces virulence products (such as the above mentioned toxins and enzymes) to aid its survival and nutrition.

Both *Porphyromonas gingivalis* and *Bacteroides forsythus* produce proteolytic enzymes known as trypsin-like serine proteases with a molecular weight of about 50 kDa. One of the proteases from *P. gingivalis* is named arg-gingipain, and one of the proteases from *B. forsythus* is a 48 kDa protease. *Actinobacillus actinomycetemcomitans*
30 produce a 116 kDa leukotoxin which is a member of the repeats-in-toxin exoprotein family of pore-forming leukotoxins and is specifically cytotoxic to human polymorphonuclear leukocytes.

In preferred embodiments, said first substance is a
35 bacterial virulence product, preferably an enzyme, such as a protease, more preferably arg-gingipain from *Porphyromonas gingivalis* and the 48 kDa protease from *Bacter-*

oides forsythus or a toxin, more preferably the leukotoxin from *Actinobacillus actinomycetemcomitans*.

The presence of virulence products may trigger an inflammatory response and the immune system of the host, thus recruiting defence system cells, e.g. polymorphonuclear (PMN) leukocytes, to the site of infection.

The leukocytes cannot cope with the high amounts of bacteria and bacterial products at the infected site and enzymes from the leukocyte granulates are released into the periodontal pocket. These enzymes, originally aimed at killing the invading bacteria, are highly destructive to the surrounding tissues. Human neutrophil elastase has been shown to degrade many of the supportive tissues surrounding a tooth. Elastase is often found bound to its protease inhibitor (α -1 antitrypsin) in the periodontal pocket. However, the protease from *P. gingivalis* has been shown to degrade the protease inhibitors α -1 antitrypsin and α -2 macroglobulin in human serum, leaving elastase in its highly destructive form in the periodontal or peri-implant pocket.

As used herein, substances "originating from the immune or inflammatory system" refers to substances that originates from cells involved in the immune or inflammatory system. Such substances may be secreted from said cells, or may originate from lysis of such cells, for example to orchestrate the immune and inflammatory response or to remodel the tissue or killing the invading bacteria.

The second substance may be a leukocyte product, such as a natural serine protease, preferably human neutrophil elastase or a cytokine, such as an interleukin preferably chosen from among interleukin-1 β , interleukin-6 and interleukin-8, or an inflammatory mediator, preferably tumour necrosis factor- α or possibly prostaglandin E₂.

Most preferably, said second substance is human neutrophil elastase.

Other substances originating from the immune or inflammatory system of the patient suitable for detection according to the present invention comprise, but are not limited to, collagenases, aminotransferases, alkaline
5 phosphatase, β -glucuronidase, dipeptidyl peptidase, neutrophil gelatinase-related lipocalin and matrix metalloproteinases.

Most preferably, said first substance is a bacterial virulence product, and said second substance is human
10 neutrophil elastase.

The co-existence of at least a first substance of bacterial origin and a second substance as defined above, preferably human neutrophil elastase, should indicate both active bacteria and an active immune or inflammatory
15 system, and this is indicative of periodontal disease. Thus it is advantageous to detect the co-existence of at least said first substances and said second substances.

In some instances, the test kit according to the present invention comprises additional detection assays
20 for the detection of additional substances in said sample, and preferably such additional substances are selected among substances originating from bacteria as defined above.

In preferred embodiments, the test kit according to the present invention said first affinity ligand comprises a first antibody exhibiting selective binding to
25 said first substance, and said second affinity ligand comprises a second antibody exhibiting a selective binding to said second substance.

30 As used herein, "antibody" refers to monoclonal antibodies, polyclonal antibodies, synthetically produced antibodies or antibody-equivalents and functional fragments thereof.

Antibodies of special interest for the present invention are antibodies that specifically binds to any of
35 the above mentioned first or second substances. Antibodies raised against human neutrophil elastase are commercial

cially available from MP Biomedicals. Antibodies against the leukotoxin from *A. actinomycetemcomitans* has been developed (Johansson et al. 2000) and antibodies against the protease from *P. gingivalis* has also been developed
5 (Nakagawa et al. 2001).

As used herein, an "assay" refers to means for detecting the presence and/or determining the amount of one or more substance(s) in a sample. The result from an analysis on an assay is a detectable response, for example as a change in colour, fluorescence, absorbance
10 and/or luminescence, a change in conductivity, a change in radioactivity etc.

Detection assays suitable for use with the test kit according to the present invention may be based on one
15 out of several immunological methods. Such methods include, but are not limited to, immunochromatographic methods, immunometric methods, immunoagglutination methods, fluoroimmunological methods, immunoluminescence methods, turbidimetric immunological methods, ELISA and
20 nephelometric methods.

In a preferred embodiment of the test kit according to the present invention, said first and second detection assays provides immunochromatographic assays, preferably embodied as a so called "strip-test", implemented as lateral flow test. There are several different variants of
25 immunochromatographic assays known to those skilled in the art. Each immunochromatographic assay preferably employs two antibodies specific for different epitopes of the substance to be detected.

Furthermore, more than one detection assay can be fitted onto one single strip-test, so that the presence of two or more substances specifically can be detected on one single strip.
30

Moreover, an immunochromatographic assay may be designed so that a predefined threshold amount of the
35 sought substance is needed in the sample to yield a positive signal, as is known to those skilled in the art.

The invention also relates to a method for diagnosing periodontal diseases and/or predicting the risk for progress of said diseases, said method comprising analyzing a sample from the oral cavity of a patient for the presence of at least a first substance originating from bacteria and the presence of a second substance originating from the immune or inflammatory system of the patient.

In the method according to the present invention, sample from the oral cavity of a patient and the first and second substances are as defined above.

Also in some instances, the diagnostic method according to the present invention further comprises the step of detecting the presence of additional substances as defined above in said sample.

In preferred embodiments of the diagnosis method according to the present invention, said first method comprises using a first antibody exhibiting selective binding of said first substance and said second method comprises using a second antibody exhibiting selective binding of said second substance, wherein said antibodies are as defined above.

Most preferably at least one of said first and second methods comprises using an immunochromatographic method.

Other detection methods suitable for use with the methods according to the present invention may be based on one out of several immunological methods. Such methods include, but are not limited to, immunochromatographic methods, immunometric methods, immunoagglutination methods, fluoroimmunological methods, immunoluminescence methods, turbidimetric immunological methods, ELISA and nephelometric methods.

Preferred embodiments

Preferably a test kit according to the invention, as illustrated in figure 1, comprises two immunochroma-

tographic detection assays 1, 2 arranged on a support 3 equipped with a sample reservoir 4 for receiving a sample. The two detection assays 1, 2 are arranged, directly or via a removably arranged separating means 5, in contact with the sample reservoir 4.

The sample reservoir 4 is preferably formed in the support material. The support 3 can be made of several different material, such as plastic, paper, carton or combinations thereof, such as paper laminated with plastic. The detection assays 1, 2 are fixed on the support 3 in such a manner that the sample receiving areas of each assay is, directly or via a removably arranged separating means 5, in contact with the sample reservoir 4. Said separating means 5 may be a removable foil covering the sample receiving areas on the assays, a dam separating the reservoir from the assays, etc.

The kit may further comprise additional buffers for dilution and adaptation of said sample for said detection assays and at least one sampling device for obtaining the sample to be analysed. The type of sampling device suitable for use may depend on the type of sample to be analysed. For example gingival crevicular fluid is a viscous fluid and can easily be collected by a small brush, a dental floss, a paper point or a disposable pipette. Other sampling devices for obtaining a saliva or mouth rinse sample are known to those skilled in the art. Preferably the buffer is kept separately from the sample reservoir in a buffer reservoir, such as a separate flask, an additional reservoir formed on the support or by providing the buffer in a puncturable bag placed in the sample reservoir.

After a sample is obtained, it is preferably mixed with buffer to obtain the pH, ionic strength and viscosity suitable for the detection assays. After mixing, the sample is transferred to the sample reservoir, and is thus, optionally by removing the separating means,

brought in contact with a receiving area of the detection assays.

Through capillary flow, the sample is allowed to migrate along a cellulose strip to another area where
5 small particles (for example colloidal gold or latex) coated with antibodies specific to one epitope of the protein (antigen) to be analysed by the detection assay is picked up by the liquid flow. Antigens present in the sample attaches to the particle bound antibodies and
10 migrate further along the cellulose strip. Further down the path of the capillary flow, irreversibly bound to the strip, is another antibody (mono- or polyclonal) that recognise another epitope of the antigen. Particles that have caught an antigen attach to the area on the strip
15 where the second antibody is bound, with the antigen sandwiched between the particle and strip bound antibodies respectively.

If enough particles get caught at the same area a visible line (test line) forms on the strip. Particles
20 that have not caught an antigen continue down the strip and some get caught on a function control line. The particles are too small to be visible to the human eye one by one. Only if enough particles get caught in the same area a visible line forms.

25 The above process takes place essentially simultaneously in both strips, whereby one result for each of the analysed substances is obtained within a few minutes.

The above described preferred embodiment of the present invention and the following experiment is intended
30 for illustrative purposes only, and shall not be interpreted as limiting the present invention. The scope of the present invention is defined by the appended claims.

Experiments

35 Material and methods

Samples for the following study were obtained from 16 volunteers who had been referred for periodontal

treatment to the Department of Periodontology, Public dental service in Kristianstad, Sweden. The age of the patients ranged from 28 to 54 years with a mean of 40. All individuals had at least three sites with a probing
 5 depth greater than or equal to 6 mm, located at separate teeth. None of the patients were medically compromised or had periodontal and/or antibiotic treatment during the preceding 6 months.

Sampling and clinical examination were preformed in
 10 duplicate, 1 week apart. The samples for enzyme analysis were collected before microbial sampling and clinical examination. The first sampling and examination was performed before any treatment was given (baseline, determination 1). The second sampling and examination
 15 occurred 6 months after completion of the first treatment (determination 2), and the third, another 6 months after the second treatment was finished (determination 3).

After baseline examination all patients received oral hygiene instructions and supra- and subgingival
 20 debridement throughout the entire dentition. At the second treatment session periodontal surgery or rescaling under anaesthesia was preformed at all sites where *P. gingivalis* constituted $\geq 0,5\%$ or *P. Intermedia* $\geq 5\%$ of the total anaerobic viable count, or *A. actinomycetemcomitans* was present in the subgingival
 25 samples. All other sites selected for the study received supragingival polish.

From each patient, samples were collected from 3 to 10 sites with initial probing depths ≥ 6 mm. The sampling
 30 area was dried and isolated with cotton rolls and, supragingivally, plaque was carefully removed with sterile curets and cotton pledgets. For the enzymes assays, three medium paper points (Johnson and Johnson, Windsor, NJ) were inserted consecutively approximately 1
 35 mm into the peridontal sulcus and left for 15 seconds. The wet part of each paper point was cut off with a sterile pair of scissors and pooled in a minisorb tube

(Nunc, Roskilde, Denmark) containing 100 μ l 0,85% NaCl. The samples were frozen immediately at -20°C and within 6 hours at -80°C , and stored until assayed. For the microbial analysis 3 paper points were inserted
5 consecutively into the periodontal pocket until resistance was met and left in place for 15 seconds. The points were pooled into a vial containing 10 glass beads, 3 mm in diameter, and 3,3 ml of VMGA III transport medium, aerobically prepared and stored. The samples were
10 processed within 24 hours. Bacteria were grown on enriched Brucella agar plates and identified by appropriate methods.

For the study of appropriate cut-off values for a diagnostic test we studied elastase from human
15 neutrophils and arg-gingipain from *P. gingivalis* using selective substrates.

The first set of the duplicate samples was used for the enzyme assays. All samples were thawed on ice and centrifuged for 3 minutes at $13,000 \times g$.
20 The enzyme substrate for determining arg-gingipain from *P. gingivalis* was N-benzoyl-L-arginine-p-nitroanilic (BAPNA) with a final concentration of 1 mM in the assay buffer containing 5 % DMSO. The assay buffer was 0.1 M Tris-HCl containing 5 mM CaCl₂, pH 7,5, with 50 mM glycyl-
25 glycine (as reported earlier by the inventors in patent US 5,981,164 gly-gly stimulates BAPNA selectively in the presence of arg-gingipain) and 5 mM L-cysteine. Ten μ L of the sample was preincubated for 15 minutes with 140 μ L of the assay buffer in the well of a 96-well microtiter
30 plate, precoated with bovine serum albumin, before 50 μ L of the substrate was added. The plate was incubated at 37°C in a humid chamber, and the release of pNA was followed spectrophotometrically by OD₄₀₅ readings, using a microtiter plate reader, every few hours from 12 to 36
35 hours. One unit of activity was equal to the amount of enzyme which cleaved 1 nmol of the substrate during one hour of incubation.

The elastase assay was preformed by adding 5 µl of CGF to a well of a 96-well microtiter plate containing 145 µl of an assay buffer (0,1 M Tris, 0,5 M NaCl at pH 7,5). After 15 minutes the reaction was started by adding 50 µl of a 2 mM solution of Methoxysuccinly-Ala-Ala-Pro-Valine-pNA in assay buffer containing 20% DMSO. The plate was incubated at 37°C in a humidified chamber. The enzymatic reaction releasing the pNA was read at OD₄₀₅ every few hours from 12 to 26 hours using a microtiter plate reader (Molecular Devices) and compared to a standard of diluted purified enzyme. The recorded readings were plotted against time and enzyme activity was calculated as DA₄₀₅/60 minutes from the linear part of the plot. The amount of elastase was presented in ng per site.

Patients with growth of *P. gingivalis* or presence of gly-gly stimulated BAPNA activity but with no growth of *A. actinomycetemcomitans* were included in the cut-off study. This criterion yielded 35 sites from 8 different patients. The determination one year after initial treatment was used in this limited study. Attachment loss as measured from the bottom of the periodontal pocket to the root-cement margin with a pressure balanced probe was used as a measurement of further progression of periodontal decease. Elastase and arg-gingipain were analysed for their ability to predict further attachment loss. Individually the elastase cut-off level were set to 20 ng per site and for arg-gingipain to 0,27 units per site.

Results

<u>Elastase</u>	Attachment loss	Attachment gain or zero
Positive test (Elastase >20 ng)	3	2
Negative test (Elastase ≤20 ng)	3	27

- 5 Elastase as a predictor of further attachment loss yielded a sensitivity of 50% and a specificity of 93%. A p-value of 0.0264* was calculated using Fisher's exact test.

<u>Arg-gingipain</u>	Attachment loss	Attachment gain or zero
Positive test (arg-gingipain >0,27 U)	6	13
Negative test (arg-gingipain ≤0,27 U)	0	16

10

Arg-gingipain as a predictor of further attachment loss yielded a sensitivity of 100% and a specificity of 55%. A p-value of 0.0216* was calculated using Fisher's exact test.

- 15 For elastase and arg-gingipain alone either the senistivity or the specificity had to be sacrificed. This prompted us to investigate the combination of the two enzymes. We investigated new cut-off levels for the two enzymes to find that the detection limit for elastase
- 20 should be 2 ng per site and 0,30 units for arg-ginipain.

<u>Combination</u>	Attachment loss	Attachment gain or zero
Positive test (Elastase >2 ng <u>and</u> arg-gingipain >0,30 U)	5	3
Negative test (Elastase ≤2 ng <u>or</u> arg-gingipain ≤0,30 U)	1	26

The combination of elastase and arg-gingipain as a
 predictor of further attachment loss yielded a
 5 sensitivity of 83% and a specificity of 90%. A p-value of
 less than 0,001*** was calculated using Fisher's exact
 test. This limited data shows that the combination of
 elastase and arg-gingipain as a marker for periodontal
 disease yields a statistically more significant test
 10 than either of the enzymes alone.

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CLAIMS

1. A test kit for detecting periodontal disease in a patient by analysing a sample from the oral cavity of the patient, wherein said kit at least comprises:

a first detection assay for detecting a first substance originating from bacteria, and

a second detection assay for detecting a second substance originating from the immune or inflammatory system of the patient.

2. A test kit according to claim 1, wherein said first detection assay comprises at least a first affinity ligand having a binding site for binding said first substance originating from bacteria, and

said second detection assay comprises at least a second affinity ligand having binding site for binding said second substance originating from the immune or inflammatory system of the patient.

3. A test kit according to claim 1 or 2, wherein said first substance is a bacterial virulence product.

4. A test kit according to claim 3, wherein said first substance is an enzyme.

5. A test kit according to claim 4, wherein said enzyme is a protease.

6. A test kit according to claim 5, wherein said protease is selected from the group consisting of arg-gingipain from *Porphyromonas gingivalis* and a 48 kDa protease from *Bacteroides forsythus*.

7. A test kit according to claim 3, wherein said first substance is a toxin.

8. A test kit according to claim 7, wherein said toxin is a leukotoxin from *Actinobacillus actinomyces-comitans*.

5 9. A test kit according to any of the preceding claims, wherein said second substance is a leukocyte product.

10 10. A test kit according to claim 9, wherein said leukocyte product is a natural serine protease.

11. A test kit according to claim 10, wherein said natural serine protease is a human neutrophil elastase.

15 12. A test kit according to any of the claims 1-8, wherein said second substance is a cytokine.

13. A test kit according to claim 12, wherein said cytokine is an interleukin.

20

14. A test kit according to claim 13, wherein said interleukin is chosen from among interleukin-1 β , interleukin-6 and interleukin-8.

25 15. A test kit according to claim 12, wherein said cytokine is an inflammatory mediator.

16. A test kit according to claim 15, wherein said inflammatory mediator is selected from the group consisting of tumour necrosis factor- α and prostaglandin E₂.

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17. A test kit according to any of the claims 2 to 16, wherein said first affinity ligand is a first antibody exhibiting selective binding of said first substance and said second affinity ligand is a second antibody exhibiting selective binding of said second substance.

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18. A test kit according to claim 17, wherein each of said first and second detection assays provides an immunochromatographic assay.

5 19. A test kit according to any of the preceding claims, further comprising a support provided with a sample reservoir for receiving said sample, wherein said first and second detection assays are arranged on said support in contact with said sample reservoir, directly
10 or via a removably arranged separating means which separates said sample reservoir from said detection assays.

20. A test kit according to any of the preceding claims, further comprising additional buffers for dilu-
15 tion and adaptation of said sample for said detection assays.

21. A test kit according to claim 20, further comprising a buffer reservoir separate from said sample reservoir.
20

22. A test kit according to any of the preceding claims, further comprising at least one sampling device for obtaining said sample.
25

23. The use of a test kit according to any of the preceding claims for detecting periodontal disease.

24. A method for diagnosing periodontal diseases
30 and/or predicting the risk for progress of said diseases, said method comprising:

analyzing a sample from the oral cavity of a patient for the presence of at least a first substance originating from bacteria and the presence of a second
35 substance originating from the immune or inflammatory system of the patient.

25. A method according to claim 24, wherein said first substance is a bacterial virulence product.

26. A method according to claims 25, wherein said first substance is an enzyme.

27. A method according to claim 26, wherein said enzyme is a protease.

28. A method according to claim 27, wherein said protease is selected from the group consisting of arg-gingipain from *Porphyromonas gingivalis* and a 48 kDa protease from *Bacteroides forsythus*.

29. A method according to claim 25, wherein said first substance is a toxin.

30. A method according to claim 29, wherein said toxin is a leukotoxin from *Actinobacillus actinomycetemcomitans*.

31. A method according to any of the claims 24-30, wherein said second substance is a leukocyte product.

32. A method according to claim 30, wherein said leukocyte product is a natural serine protease.

33. A method according to claim 32, wherein said natural serine protease is a human neutrophil elastase.

34. A method according to any of the claims 24-30, wherein said second substance is a cytokine.

35. A method according to claim 36, wherein said cytokine is an interleukin.

36. A method according to claim 35, wherein said interleukin is chosen from among interleukin-1 β , interleukin-6 and interleukin-8.

5 37. A method according to claim 36, wherein said cytokine is an inflammatory mediator.

10 38. A method according to claim 37, wherein said inflammatory mediator is selected from the group consisting of tumour necrosis factor- α and prostaglandin E₂.

15 39. A method according to any of the claims 24-38, wherein said analyzing comprises analyzing said sample with a first method that selectively detects the presence of said first substance and a second method that selectively detects the presence of said second substance.

20 40. A method according to claim 39, wherein said first method comprises using a first antibody exhibiting selective binding of said first substance and wherein said second method comprises using a second antibody exhibiting selective binding of said second substance.

25 41. A method according to claim 40, wherein at least one of said first and second methods comprises using an immunochromatographic assay.

30

Abstract

Test kit for diagnosing periodontal disease in a patient by analysing a sample from the oral cavity of the patient. The test kit comprises at least a first detection assay for detection of a first substance originating from bacteria and at least a second detection assay for detection of a second substance originating from the immune or inflammatory system of the patient.

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Figure 1 is to be published on the front page.

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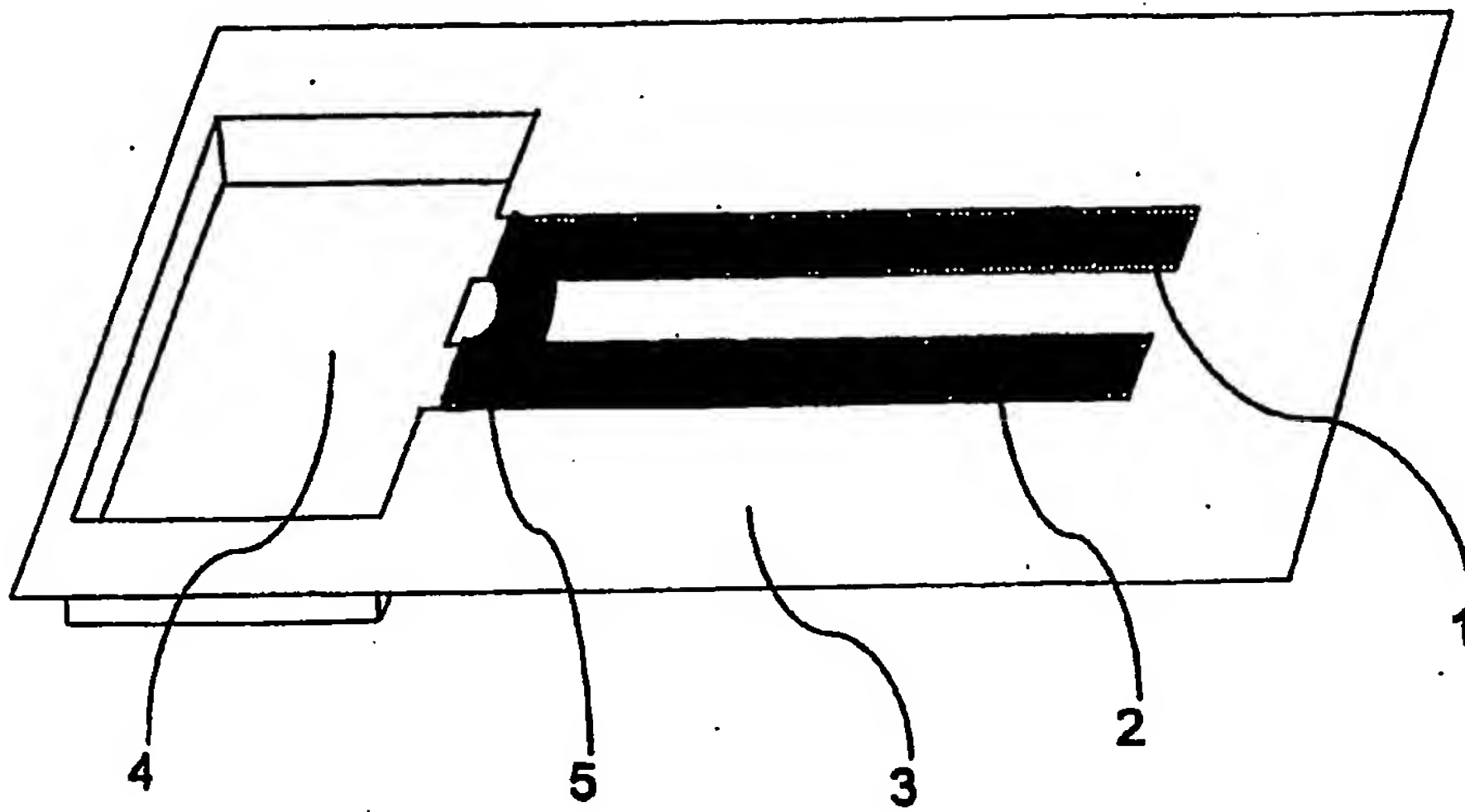


Fig. 1

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Date of mailing (day/month/year) 08 February 2005 (08.02.2005)	
Applicant's or agent's file reference 21017477	IMPORTANT NOTIFICATION
International application No. PCT/SE04/001883	International filing date (day/month/year) 16 December 2004 (16.12.2004)
International publication date (day/month/year)	Priority date (day/month/year) 30 January 2004 (30.01.2004)
Applicant BENGTTSSON, Thomas et al	

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<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
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